



# APPENDIX I

Effects of Unfractionated Heparin, Low  
Molecular Weight, Synthetic Polysaccharides  
and Pentasaccharide on CD62-mediated  
Platelet-neutrophil Adhesion

**EFFECTS OF UNFRACTIONATED HEPARIN, LOW MOLECULAR WEIGHT  
HEPARIN, SYNTHETIC POLYSACCHARIDES AND PENTASACCHARIDE ON  
CD62-MEDIATED PLATELET-NEUTROPHIL ADHESION.**

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**I hereby certify that the experimental studies described and the analyses presented in  
this report were conducted by me and/or under my supervision.**

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## I. BACKGROUND

Platelet satellitism is due to the adhesion of activated platelets to leukocytes. This cellular interaction is classical in various pathological settings such as inflammation or prothrombotic states. This phenomenon is mediated by both P-selectin (CD62) and its counterreceptor CD15 on Polymorphonuclear leucocytes (PMNL) (Hamburger, Blood 1990). It has been reported that unfractionated heparin (UFH) and low molecular weight heparin (LMWH) such as enoxaparin inhibit this CD62-induced rosette formation (Dagenais et al, 1996, 1997; Libersan et al, 1998). Spangenberg et al have indicated that  $\alpha_{2b}\beta_3$  (CD41a) seems also involved in this process in human (1993). Complex interactions between neutrophils and platelets, two major cellular elements in vascular compartment, modulate the response to vascular injury or inflammation.

Cellular accumulation is decreased in a concentration-dependent manner by anticoagulation limiting infarct size after reperfusion in experimental models (Dagenais et al, Faseb, 1997). When *in vitro* rosettes were prepared in aqueous buffer enoxaparin and UFH were equipotent. However, in a plasma milieu, UFH lost its efficacy to block CD62 while enoxaparin remains able to interfere significantly with P-selectin mediated cell-adhesion. This effect was not related to their anti-Xa or anti-IIa activities.

Recently, Myers et al have shown that specific antibody against P-selectin is able to prevent venous thrombosis in an animal model, underlying the importance of the inflammatory response mediated by P-selectin in thrombotic processes (2002).

Furthermore, Aggarwal et al, reported recently that platelet reactivity was significantly greater in blood exposed to UFH than in blood exposed to enoxaparin with respect to P-selectin expression in response to ADP *in vitro* stimulation (Aggarwal et al, J Thromb Thrombolys, 2002).

## II. AIMS OF THE STUDY

Using flow cytometry analysis, we have compared the *in vitro* effect of various polysaccharides species on this P-selectin-mediated leucocyte association in normal citrated human blood samples.

First, with a different experimental approach, we will confirm the literature data concerning the inhibitory capacity of heparin on this cell-cell interaction.

Second, using flow conditions and various milieus, we will determine if there is a different potency between these compounds.

The main objective of this study is also to obtain a better understanding of the existing relation between "biological effect and physical structure" of oligosaccharides on this P-selectin mediated phenomenon.

## II. MATERIALS AND METHODS

Our objective is to compare the effects of these different compounds on complexes formation in a purified milieu (using washed platelets) and autologous whole blood. Platelets and polymorphonuclear leucocytes were isolated from healthy donors (citrated blood).

In a first step, we determined the optimal conditions leading to CD62-mediated leucocyte complexes through platelet activation in a purified milieu. We have used TRAP to activate platelets and to trigger P-selectin secretion. Leucocytes were isolated by a classical ficoll procedure. For whole blood experiments, we have incubated oligosaccharides during 15 minutes at 37°C before adding platelet agonist with CD41 and CD16 markers.

In a second step, we have screened the potential of various polysaccharides to interfere with this adhesion process involving neutrophils and platelets. Using the TRAP at the same concentration (5  $\mu$ M), we have activated whole citrated blood cells and fixed generated complexes with PFA (1%).

Thus, we have determined by flow cytometry the ability of these different polysaccharides to inhibit neutrophils complexes formation mediated by P-selectin (CD62-P).

The experimental work was done as follows :

We used a FACScan apparatus (Beckton Dickinson).

Heparin and various polysaccharides were obtained from Aventis Pharma. TRAP and PBS buffer were obtained from Neosystem and Sigma. Recombinant human P-selectin was purchased from Calbiochem and used at a saturating rate. Specific monoclonal antibodies were from Becton Dickinson. These antibodies were coupled to fluorescent marker (FITC fluorescein isothiocyanate or PE, phycoerethrin). We have used an anti-CD62-P (anti-GMP140, anti-P-selectin) and an anti-CD41 (anti-GPIIb) for platelet analysis and an anti-

CD16 (against neutrophils) for leucocyte analysis. We have compared the inhibitory effect on complexes formation determined by the modification of the percentage of both CD62 and CD16 expression.

All the polysaccharides were tested at two different concentrations (100 and 10 µg/ml) to determine a structure-function analysis or a dose dependent process.

In addition to unfractionated heparin (Choay®), pentasaccharide (Arixtra®) and Enoxaparin (Lovenox®), thirteen other products have been studied:

**Species Nomenclature (< 7% 1, 6 Anhydro)**

	<b>DIA 2844 (&lt; 7% 1, 6 Anhydro)</b>	<b>WSD3093 (15-25% 1, 6 Anhydro)</b>
<b>&lt; HEXDECAASACCHARIDE</b>	<b>C<sub>2</sub></b>	<b>C<sub>8</sub></b>
<b>≥ HEXADECASACCHARIDE</b>	<b>C<sub>3</sub></b>	<b>C<sub>9</sub></b>
<b>HEXASACCHARIDE</b>	<b>C<sub>4</sub></b>	<b>C<sub>10</sub></b>
<b>OCTASACCHARIDE</b>	<b>C<sub>5</sub></b>	<b>C<sub>11</sub></b>
<b>DECASACCHARIDE</b>	<b>C<sub>6</sub></b>	<b>C<sub>12</sub></b>
<b>DODECASACCHARIDE</b>	<b>C<sub>7</sub></b>	<b>C<sub>13</sub></b>

**C1 = Enoxaparin Polysaccharides**

Flow cytometric analysis of combined fluorescence, CD41 and CD16, is used to evaluate the potential inhibition of P-selectin-mediated complexes formation. We have determined the complexes formation after in vitro TRAP activation of platelets. EDTA was used to inhibit this complexes formation confirming that it is a calcium dependent process. The results are

expressed as a mean of fluorescent-positive percentage inhibition compared to the control value. This screening was done on three different healthy volunteers.

### **III. RESULTS**

#### **A. CD62-MEDIATED COMPLEXES FORMATION IN PURIFIED MILIEU**

Taking into account the literature data, we have determined the optimal experimental conditions leading to obtain about 50% (30 to 70%) of neutrophils-platelets complexes in a purified system.

After activation with TRAP during 5 minutes and fixation with paraformaldehyde 1%, citrated platelets were washed. In a second step, we isolated autologous neutrophils using a classical ficoll technique. After washing twice in PBS buffer, we adjusted this cell suspension to maintain a determined ratio between neutrophils and platelets (1/50).

Then, we incubated fixed washed platelets and heparin or other oligosaccharides during 20 min at 37°C. After neutrophils addition, another incubation period was done (30 min, 37°C). Fluorescent antibodies binding was analyzed by flow cytometry after another incubation of 15 min at room temperature.

The results of this method are shown in Table 1.

**Table 1. Mean Inhibition of Platelet-Leucocyte Complexes Formation (%) in Purified Milieu**

<b>Polysaccharide ug/mL</b>	<b>10</b>	<b>100</b>
<b>Pentasaccharide</b>	<b>-</b>	<b>0</b>
<b>UFH</b>	<b>7</b>	<b>29</b>
<b>Enoxaparin</b>	<b>11</b>	<b>26</b>
<b>C1</b>	<b>8</b>	<b>24</b>
<b>C2</b>	<b>10</b>	<b>36</b>
<b>C8</b>	<b>5</b>	<b>26</b>
<b>C3</b>	<b>11</b>	<b>39</b>
<b>C9</b>	<b>8</b>	<b>40</b>
<b>C4</b>	<b>12</b>	<b>27</b>
<b>C10</b>	<b>2</b>	<b>8</b>
<b>C5</b>	<b>11</b>	<b>28</b>
<b>C11</b>	<b>9</b>	<b>25</b>
<b>C6</b>	<b>7</b>	<b>21</b>
<b>C12</b>	<b>10</b>	<b>38</b>
<b>C7</b>	<b>10</b>	<b>27</b>
<b>C13</b>	<b>12</b>	<b>39</b>

**EDTA Alone = 95% Inhibition**

## B. CD62-MEDIATED COMPLEXES FORMATION IN WHOLE CITRATED BLOOD

Using autologous whole citrated blood, the same procedure was followed concerning oligosaccharides exposure and antibodies binding.

All the results are summarised in Table 2.

**Table 2. Mean Inhibition of Platelet-Leucocyte Complex Formation (%) in Whole Citrated Blood**

Polysaccharide Concentration (ug/mL)	10	100
Pentasaccharide	-	0
UHF	2	20
Enoxaparin	15	35
C1	13	28
C2	10	36
C8	4	11
C3	10	37
C9	10	41
C4	10	34
C10	2	19
C5	2	18
C11	4	13
C6	7	22
C12	8	24
C7	8	23
C13	15	47

EDTA Alone =97% Inhibition

## V. DISCUSSION AND PERSPECTIVES

In presence of oligosaccharides, we have effectively observed a partial inhibition of platelet-leucocyte complexes formation mediated by P-selectin which is reported by others (Dagenais et al, 1997). This inhibitory effect leads to almost 30% reduction of the generation of these complexes. Oligosaccharides inhibitory effect on P-Selectin-mediated hetero-complexes formation is a dose dependent process with a stronger effect at the higher concentration.

In a purified milieu, unfractionated heparin (UFH) seems as effective as enoxaparin as reported in previous studies with another type of measurement (Dagenais et al, 1997). Not unexpectedly, the profile induced by C1 is similar to commercial Enoxaparin (Lovenox®).

In whole citrated blood, UFH remains able to inhibit P-selectin mediation for complexes formation only at high concentration. For enoxaparin, this inhibitory effect is still significant at 100 µg/ml as well as at 10 µg/ml. Thus, enoxaparin seems more effective than UFH in whole blood analysis while their inhibitory effect seems comparable in a purified milieu.

Despite a high *in vitro* concentration, Pentasaccharide did not modify the P-selectin-mediated complexes formation neither in a purified nor in a whole blood milieus.

Regarding the other studied oligosaccharides, we observed a significant inhibitory effect only at high concentration. Their inhibitory effect was generally more pronounced in a purified milieu than in citrated whole blood.

In fact, regarding hexadecasaccharides products (C2/C8; C3/C9), it is interesting to note that their inhibitory profile is similar in both milieus and is not significantly different from that of enoxaparin. Furthermore, the C2 effect seems more pronounced than that of C8 but this is not the case of C3 and C9. So there is no evident difference regarding DIA or WSD structure of these oligosaccharides regarding their inhibitory profile.

Concerning **hexasaccharides (C4/C10)**, **decasaccharides (C6/C12)** and **dodecasaccharides (C7/C13)**, a greater inhibitory effect in whole blood than in purified milieu was not observed. There is no difference regarding the DIA or WSD structure. Finally, **octosaccharides (C5/C11)** seem a little less effective in whole blood than in purified milieu.

**Table 3. Summary of oligosaccharides inhibitory effect : comparison between DIA and WSD structures**

C2 > C8	C3 $\approx$ C9
C4 > C10	C5 $\approx$ C11
C13 > C7	C6 $\approx$ C12

In previous studies, the authors suggested that low molecular weight heparin was more bioavailable than unfractionated heparin to explain a similar inhibitory effect in purified conditions and a weaker potency of unfractionated heparin in plasma (Dagenais et al, 1997).

We decided to use whole blood flow cytometric analysis to be closer to the *in vivo* conditions: presence of all native blood cells and flow conditions. Thus, in our experimental conditions, we observed such a difference. In fact, UFH was a weaker inhibitor of P- selectin mediated aggregating process in whole blood milieu. Interestingly, pentasaccharide was unable to inhibit this process.

At the same concentration, this inhibitor potency of enoxaparin was greater than that of UFH in whole citrated blood. This inhibitory profile depended on oligosaccharide size and DIA or WSD nature of their structure. In fact, the greater effect was observed with oligosaccharides close to enoxaparin size whatever their WSD or DIA structure.

All these results demonstrate that heparin inhibitory effect is mediated by a direct interaction with P-selectin before its adhesion to the cellular membrane. We postulate that heparin needs

to interfere with the monomeric form of P-selectin leading to the inhibition of leucoaggregates formation. After adhesion, P-selectin bound to platelets or other plasma proteins seems less sensitive to the inhibition by oligosaccharides. This result requires further experiments.

### **Bibliography**

- Hamburger SA. McEver RP : GMP-140 mediates adhesion of stimulated platelets to neutrophils. *Blood*, 1990, 75, 550
- Libersan D et al. The low molecular weight heparin, enoxaparin, limits infarct size at reperfusion in the dog. *Cardiovasc Res.* 1998;37:656.
- Dagenais et al. Comparative study of unfractionated and a low molecular weight heparin (enoxaparin) as P-selectins inhibitors. *Faseb Journal*, 1997, 11, A311.
- Spangenberg et al. The platelet glycoprotein IIbIIIa complex is involved in the adhesion of activated platelets to leukocytes. *Thromb Haemost*, 1993, 70, 514
- Myers D, et al. New and effective treatment of experimentally induced venous thrombosis with anti-inflammatory rPSGL-Ig. *Thromb Haemost*. 2002;87:374.

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